Violae Herba

Figure 1 A photograph of Violae Herba

A. Violae Herba B. Upper (left) and lower (right) surface of leaf C. Capsule

1. NAMES

Official Name: Violae Herba

Chinese Name: 紫花地丁

Chinese Phonetic Name: Zihuadiding

2. SOURCE

Violae Herba is the dried herb of *Viola yedoensis* Makino (Violaceae). The whole plant is collected in spring and autumn, foreign matter removed, then dried under the sun to obtain Violae Herba.

3. DESCRIPTION

Usually crumpled into masses. The main roots long-conical, 1-3.5 mm in diameter, pale yellowish-brown, with fine longitudinal wrinkles. Leaves basal, greyish-green to brownish-green, lanceolate or ovate-lanceolate when intact and flattened, 1.6-8.6 cm long, 0.6-4.7 cm wide; apex obtuse, base truncate or somewhat cordate, margin obtusely serrate, both surfaces pubescent; petioles slender, 2.2-13.5 cm long, with distinct narrow wings at the upper part. Capsules loculicidally 3-valved; seeds numerous, pale brownish-yellow. Odour slight; taste slightly bitter and sticky (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Root: Cork consists of several layers of cells. Cortex broad, parenchymatous cells subrounded, filled with starch granules; scattered with mucilage cells and clusters of calcium oxalate. Phloem relatively broad. In the xylem, vessels usually scattered singly or in groups, arranged radially [Fig. 2 (i)].

Leaf: The upper epidermal cells relatively large, tangentially elongated. The lower epidermal cells relatively small. The mesophyll consists of 1 to 2 layers of palisade cells and several layers of spongy cells, some cells contain clusters of calcium oxalate. Vascular bundle of the midrib collateral. Collenchyma present on the inner sides of the upper and lower epidermis of the midrib [Fig. 2 (ii)].

Powder

Colour greyish-green to brownish-green. Clusters of calcium oxalate 4-68 μm in diameter; polychromatic under the polarized microscope. Non-glandular hairs unicellular, 56-366 μm long (occasionally up to 408 μm), 11-65 μm in diameter, with short cuticular striations. Upper epidermal cells relatively large, subpolygonal in surface view, anticlinal wall beaded. Lower epidermal cells subpolygonal in surface view, anticlinal wall slightly wavy, sometimes beaded, stomata anisocytic, numerous. Sclerenchymatous cells of testa slender, pits distinct. Cork cells pale brownish-yellow, subsquare or subpolygonal in surface view. Starch granules scattered, simple starch granules subrounded, 1-25 μm in diameter, hilum distinct, dotted, slit-shaped, V-shaped or stellate, striations indistinct; black and cruciate-shaped under the polarized microscope; compound starch granules composed of 2-9 units. Vessels mainly spiral and reticulate, 4-41 μm in diameter (Fig. 3).

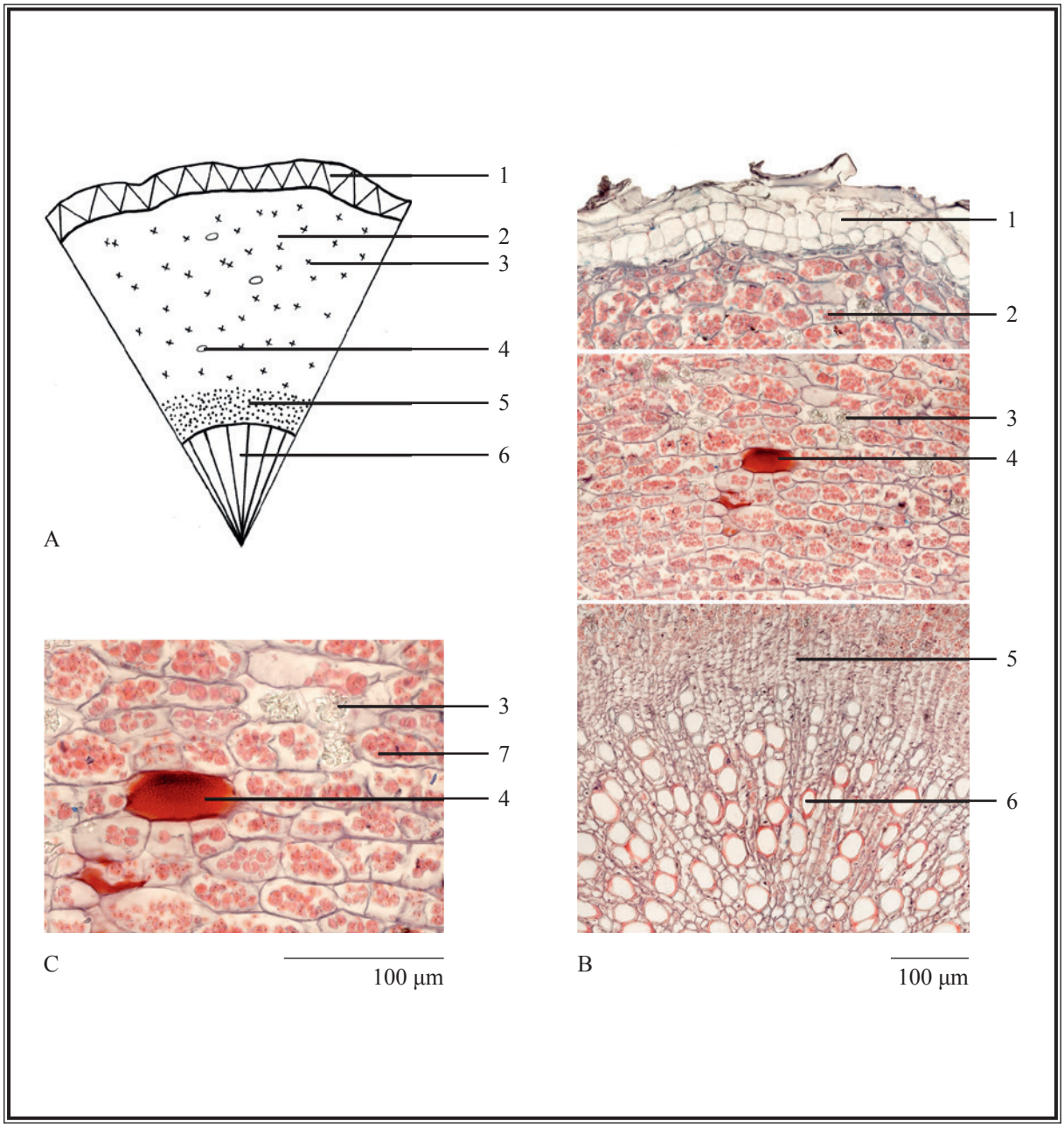

Figure 2 (i) Microscopic features of transverse section of root of Violae Herba

A. Sketch B. Section illustration
C. Clusters of calcium oxalate, mucilage cell and starch granules in cortex

1. Cork 2. Cortex 3. Cluster of calcium oxalate 4. Mucilage cell 5. Phloem
6. Xylem 7. Starch granule

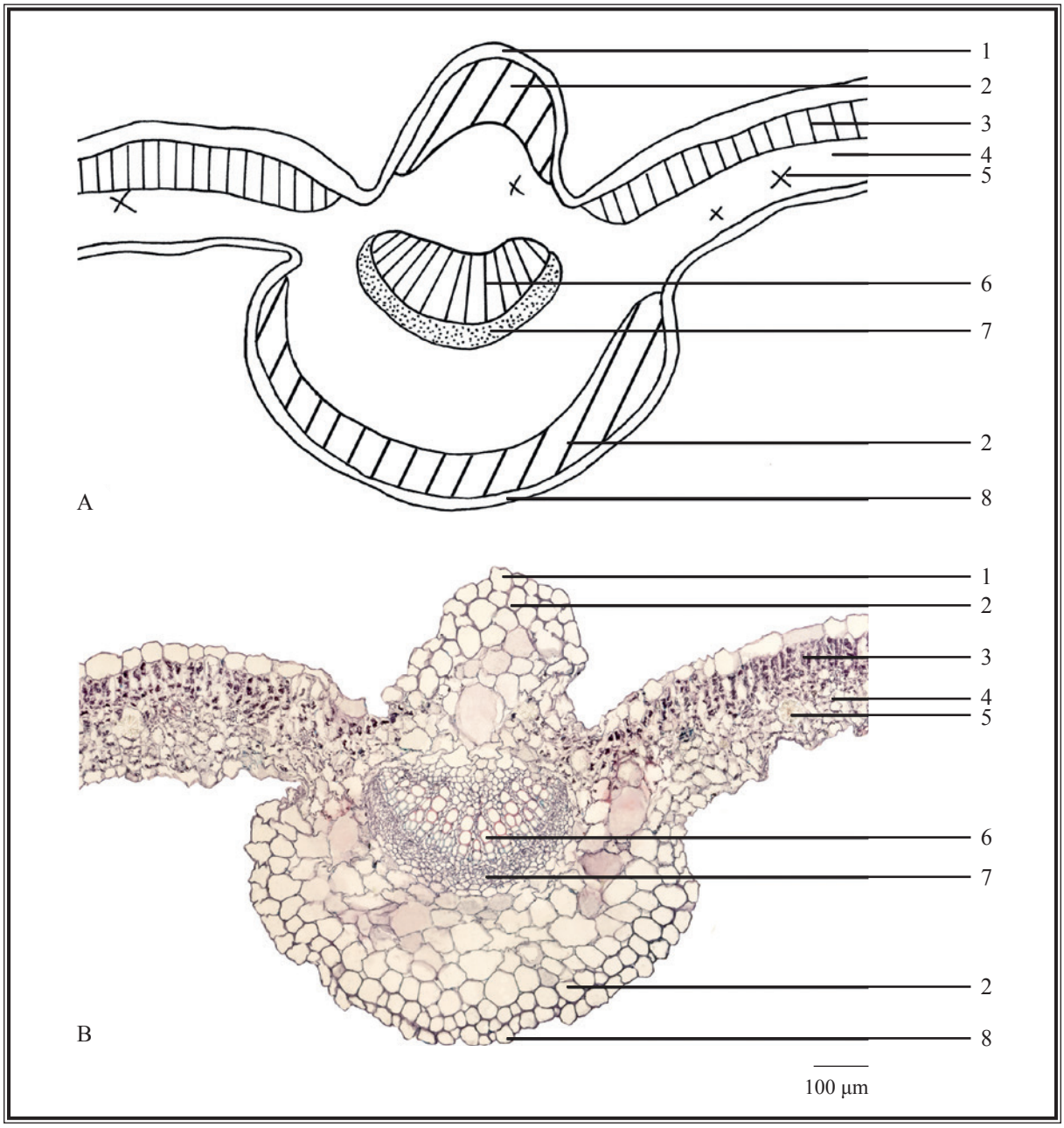

Figure 2 (ii) Microscopic features of transverse section of leaf of Violae Herba

A. Sketch of midvein B. Section illustration of midvein

1. Upper epidermis 2. Collenchyma 3. Palisade tissue 4. Spongy tissue
5. Cluster of calcium oxalate 6. Xylem 7. Phloem 8. Lower epidermis

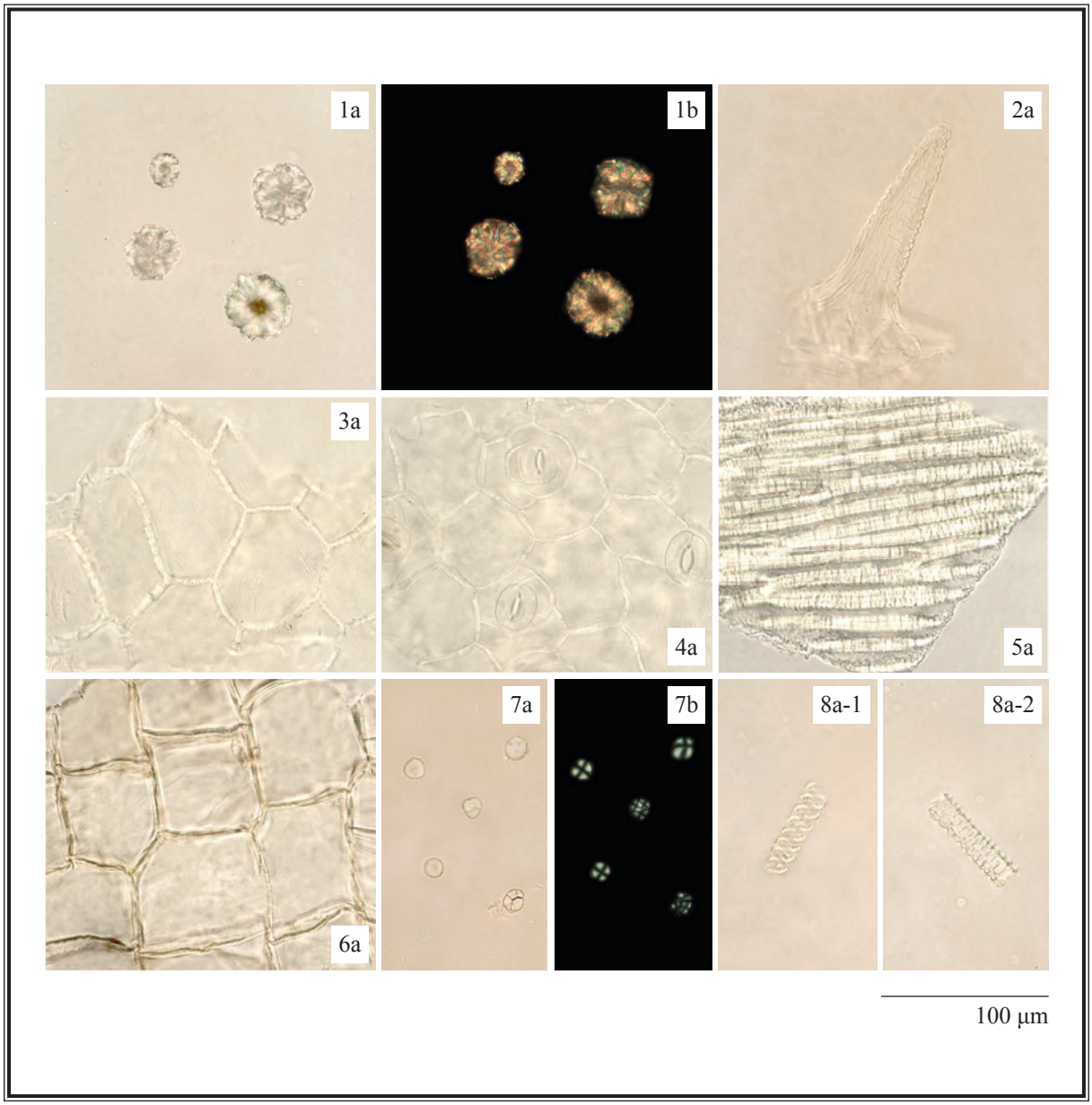

Figure 3 Microscopic features of powder of Violae Herba

1. Clusters of calcium oxalate 2. Non-glandular hair 3. Upper epidermal cells
4. Lower epidermal cells 5. Sclerenchymatous cells of testa 6. Cork cells
7. Starch granules 8. Vessels (8-1 spiral vessel, 8-2 reticulate vessel)

a. Features under the light microscope b. Features under the polarized microscope

4.2 Thin-Layer Chromatographic Identification *[Appendix IV(A)]*

Standard solution

Esculetin standard solution

Weigh 1.0 mg of esculetin CRS (Fig. 4) and dissolve in 1 mL of ethanol.

Developing solvent system

Prepare a mixture of n-butyl acetate, methanol and formic acid (15:1:0.5, v/v).

Test solution

Weigh 1.0 g of the powdered sample and place it in a 15-mL centrifuge tube, then add 10 mL of methanol. Sonicate (140 W) the mixture for 30 min. Centrifuge at about 2800 × *g* for 10 min. Filter through a 0.45-μm nylon filter.

Procedure

Carry out the method by using a HPTLC silica gel F_{254} plate, a twin trough chamber and a freshly prepared developing solvent system as described above. Apply separately esculetin standard solution and the test solution (1 μL each) to the plate. Before the development, add the developing solvent to one of the troughs of the chamber and place the HPTLC plate in the other trough. Cover the chamber with a lid and let equilibrate for about 15 min. Carefully tilt the chamber to allow sufficient solvent to pass from the trough containing the solvent to the other containing the HPTLC plate for development. Develop over a path of about 6 cm. After the development, remove the plate from the chamber, mark the solvent front and dry in air. Examine the plate under UV light (366 nm). Calculate the R_f value by using the equation as indicated in Appendix IV (A).

For positive identification, the sample must give spot or band with chromatographic characteristics, including the colour and the R_f value, corresponding to that of esculetin.

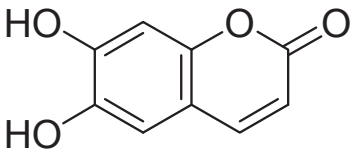

Figure 4 Chemical structure of esculetin

4.3 High-Performance Liquid Chromatographic Fingerprinting *(Appendix XII)*

Standard solution

Esculetin standard solution for fingerprinting, Std-FP (50 mg/L)

Weigh 0.5 mg of esculetin CRS and dissolve in 10 mL of ethanol (70%).

Test solution

Weigh 0.2 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of ethanol (70%). Sonicate (270 W) the mixture for 30 min. Centrifuge at about 3500 × *g* for 10 min. Transfer the supernatant to a 25-mL volumetric flask. Wash the residue with ethanol (70%). Centrifuge at about 3500 × *g* for 10 min. Combine the supernatants and make up to the mark with ethanol (70%). Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (340 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The flow rate is about 1.0 mL/min. Programme the chromatographic system as follows (Table 1) –

Table 1 Chromatographic system conditions

Time (min)	0.1% Phosphoric acid (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 15	95 → 85	5 → 15	linear gradient
15 – 40	85 → 74	15 → 26	linear gradient
40 – 60	74 → 95	26 → 5	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 µL of esculetin Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak area of esculetin should not be more than 5.0%; the RSD of the retention time of esculetin peak should not be more than 2.0%; the column efficiency determined from esculetin peak should not be less than 50000 theoretical plates.

The *R* value between peak 2 and the closest peak in the chromatogram of the test solution should not be less than 1.0 (Fig. 5).

Procedure

Separately inject esculetin Std-FP and the test solution (10 µL each) into the HPLC system and record the chromatograms. Measure the retention time of esculetin peak in the chromatogram of esculetin Std-FP and the retention times of the five characteristic peaks (Fig. 5) in the chromatogram of the test solution. Identify esculetin peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of esculetin Std-FP. The retention times of esculetin peaks from the two chromatograms should not differ by more than 2.0%. Calculate the RRTs of the characteristic peaks by using the equation as indicated in Appendix XII.

The RRTs and acceptable ranges of the five characteristic peaks of Violae Herba extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the five characteristic peaks of Violae Herba extract

Peak No.	RRT	Acceptable Range
1	0.75	± 0.03
2 (marker, esculetin)	1.00	-
3	1.26	± 0.03
4	1.29	± 0.03
5	1.49	± 0.03

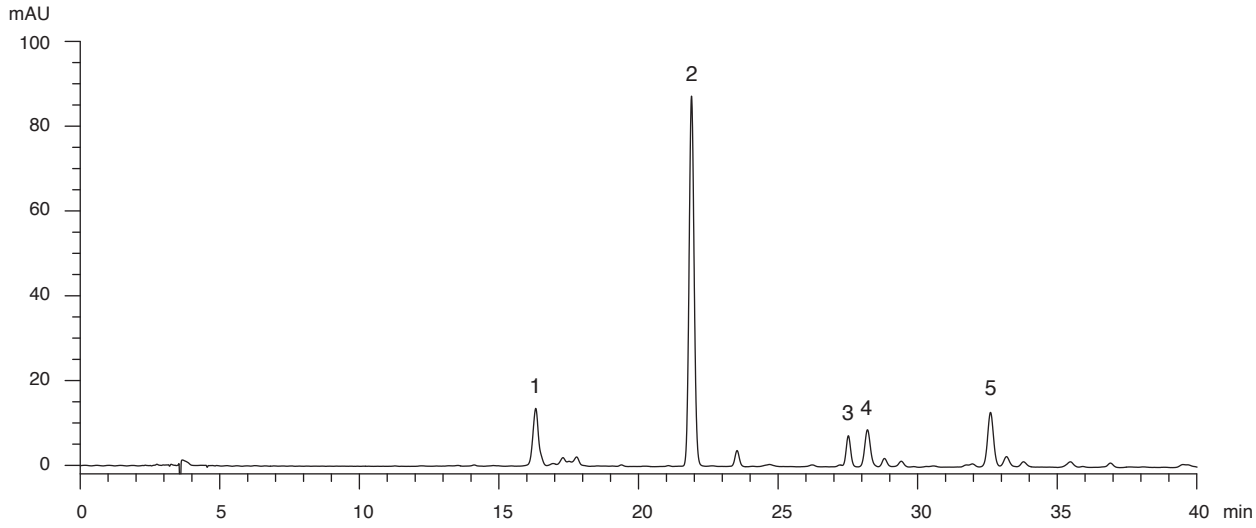

Figure 5 A reference fingerprint chromatogram of Violae Herba extract

For positive identification, the sample must give the above five characteristic peaks with RRTs falling within the acceptable range of the corresponding peaks in the reference fingerprint chromatogram (Fig. 5).

5. TESTS

5.1 Heavy Metals *(Appendix V)*: meet the requirements.

5.2 Pesticide Residues *(Appendix VI)*: meet the requirements.

5.3 Mycotoxins *(Appendix VII)*: meet the requirements.

5.4 Sulphur Dioxide Residues *(Appendix XVII)*: meet the requirements.

5.5 Foreign Matter *(Appendix VIII)*: not more than 4.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 24.0%.
Acid-insoluble ash: not more than 8.5%.

5.7 Water Content *(Appendix X)*

Oven dried method: not more than 13.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (cold extraction method): not less than 16.0%.
Ethanol-soluble extractives (cold extraction method): not less than 13.0%.

7. ASSAY

Carry out the method as directed in Appendix IV(B).

Standard solution

Esculetin standard stock solution, Std-Stock (1000 mg/L)

Weigh accurately 1.0 mg of esculetin CRS and dissolve in 1 mL of ethanol (70%).

Esculetin standard solution for assay, Std-AS

Measure accurately the volume of the esculetin Std-Stock, dilute with ethanol (70%) to produce a series of solutions of 5, 10, 20, 50, 100 mg/L for esculetin.

Test solution

Weigh accurately 0.2 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of ethanol (70%). Sonicate (270 W) the mixture for 30 min. Centrifuge at about 3500 × *g* for 10 min. Transfer the supernatant to a 25-mL volumetric flask. Wash the residue with ethanol (70%). Centrifuge at about 3500 × *g* for 10 min. Combine the supernatants and make up to the mark with ethanol (70%). Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (340 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The flow rate is about 1.0 mL/min. Programme the chromatographic system as follows (Table 3) –

Table 3 Chromatographic system conditions

Time (min)	0.1% Phosphoric acid (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 15	95 → 85	5 → 15	linear gradient
15 – 40	85 → 74	15 → 26	linear gradient
40 – 60	74 → 95	26 → 5	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 µL of esculetin Std-AS (20 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of esculetin should not be more than 5.0%; the RSD of the retention time of esculetin peak should not be more than 2.0%; the column efficiency determined from esculetin peak should not be less than 50000 theoretical plates.

The *R* value between esculetin peak and the closest peak in the chromatogram of the test solution should not be less than 1.5.

Calibration curve

Inject a series of esculetin Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of esculetin against the corresponding concentrations of esculetin Std-AS. Obtain the slope, y-intercept and the r^2 value from the 5-point calibration curve.

Procedure

Inject 10 µL of the test solution into the HPLC system and record the chromatogram. Identify esculetin peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of esculetin Std-AS. The retention times of esculetin peaks from the two chromatograms should not differ by more than 2.0%. Measure the peak area and calculate the concentration (in milligram per litre) of esculetin in the test solution, and calculate the percentage content of esculetin in the sample by using the equations as indicated in Appendix IV(B).

Limits

The sample contains not less than 0.20% of esculetin ($C_9H_6O_4$), calculated with reference to the dried substance.